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Evaluation of Antiproliferative Potency and Induced Biochemical Parameters of Some Coumarin Derivatives towards Human Breast and Liver Cancer Cell Lines

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Abstract For monitoring growth inhibition of human cancer cells, a series of novel derivatives of coumarin possessing a broader spectrum of antitumor activity and fewer toxic side effects than traditional anticancer drugs have been studied.

Eight selected coumarin derivatives (compounds 2-3b-5-6b-8b-10b-11a and 12) were subjected to a screening system for investigation of their antitumor potency against breast cancer (MCF7) and liver (HEPG2) cell lines. Moreover, the biochemical effects of the selected coumarin derivatives on some enzymes such as aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP), in addition to albumin, globulins, creatinine, total lipids, cholesterol, triglycerides and bilirubin in serum of mice were studied in comparison to 5-Flurouracil and Doxorubicin.

The antitumor activity results indicated that the selected coumarin derivatives showed growth inhibition activity against the tested cell lines but with varying intensities extents in comparison to the known anticancer drugs: 5-Flurouracil and Doxorubicin. Moreover, compounds 8b, 11a, and 6b showed the highest cytotoxic activity (IC_{50} equals 0.9, 1.28 and 1.94 µg/ml respectively). Results of the biochemical investigations indicated that 5-Flurouracil and Doxorubicin caused significant changes in the level of all parameters tested while treatment with the selected compounds showed slight, moderate or no significant changes.

In this study, we have identified coumarin derivatives as a novel class of compounds related to anti-proliferative activity. The lead compounds 8b, 11a were the more potent in the biological assay employed (e.g.: improved growth inhibition potential as compared to the reference anticancer drugs).

These experimental findings may provide support for the use of these novel compounds as anticancer agents.

Keywords Cytotoxic activity, Coumarin derivatives, Breast (MCF7), liver (HEPG2) Cancer cell lines

Introduction

Coumarin derivatives have attracted intense interest in recent years due to their broad spectrum of biological and pharmacological activities [1,2]. Many of coumarin derivatives have been reported to be active as antibacterial [3-5], antifungal [6,7], anticoagulant [8], anti-inflammatory [9], anti-HIV [10-13], anti-angiogenesis [14] and antitumors [15-18]. Since some coumarin derivatives are inhibitors of aromatase enzyme so they may be useful in suppressing aromatase and estrogen receptor-positive breast cancer [19-21].

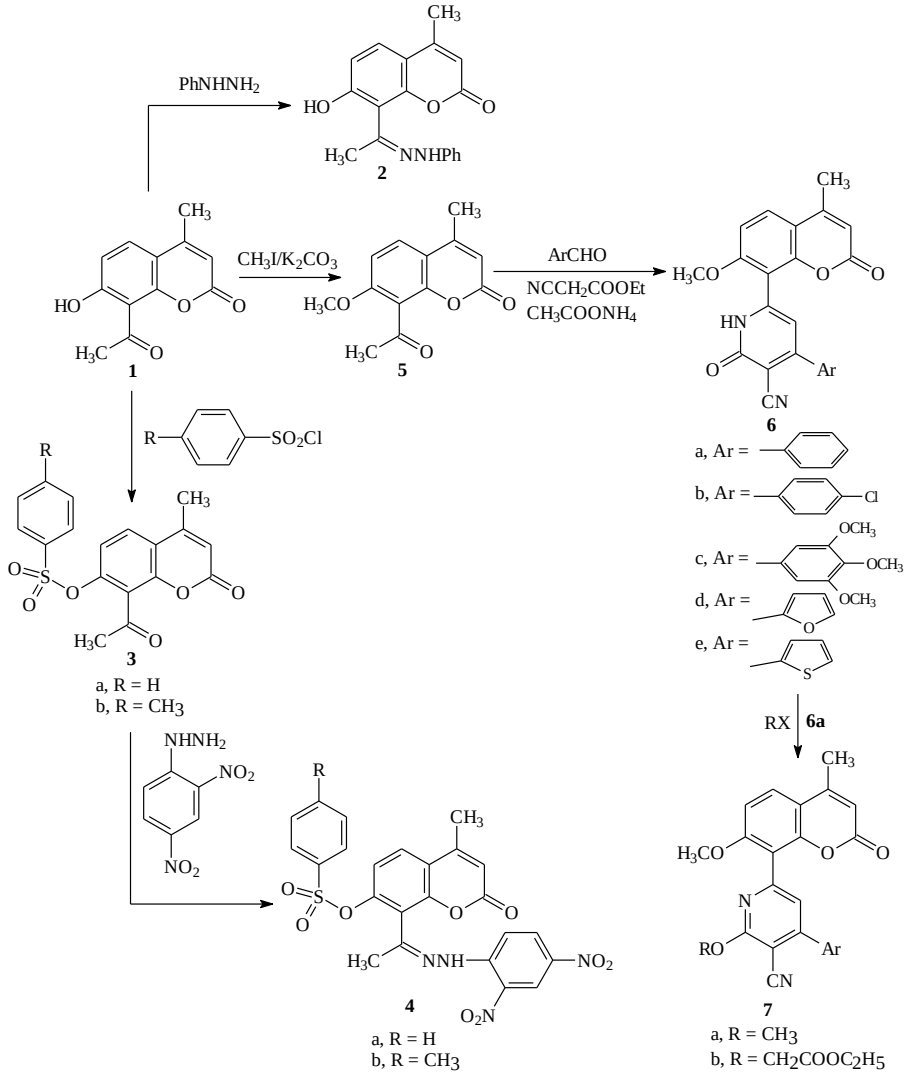
Based on these findings the present work aimed to synthesize a new group of coumarin compounds incorporated with different heterocycles as a trial that the resulting compounds would have better biological activity as

antiproliferative agents in the field of breast cancer. Since all the selected coumarin derivatives were soluble in DMSO at concentrations high enough to allow cell experiments, the in vitro biological activity of these compounds was evaluated by their growth-inhibitory potency in MCF-7 (estrogen receptor positive breast cancer cells), and liver HEPG2 cancer cell lines. The cytotoxic potency and biochemical analysis of the selected compounds (2, 3b, 5, 6b, 8b, 10b, 11a and 12) were discussed in the current study.

Experimental

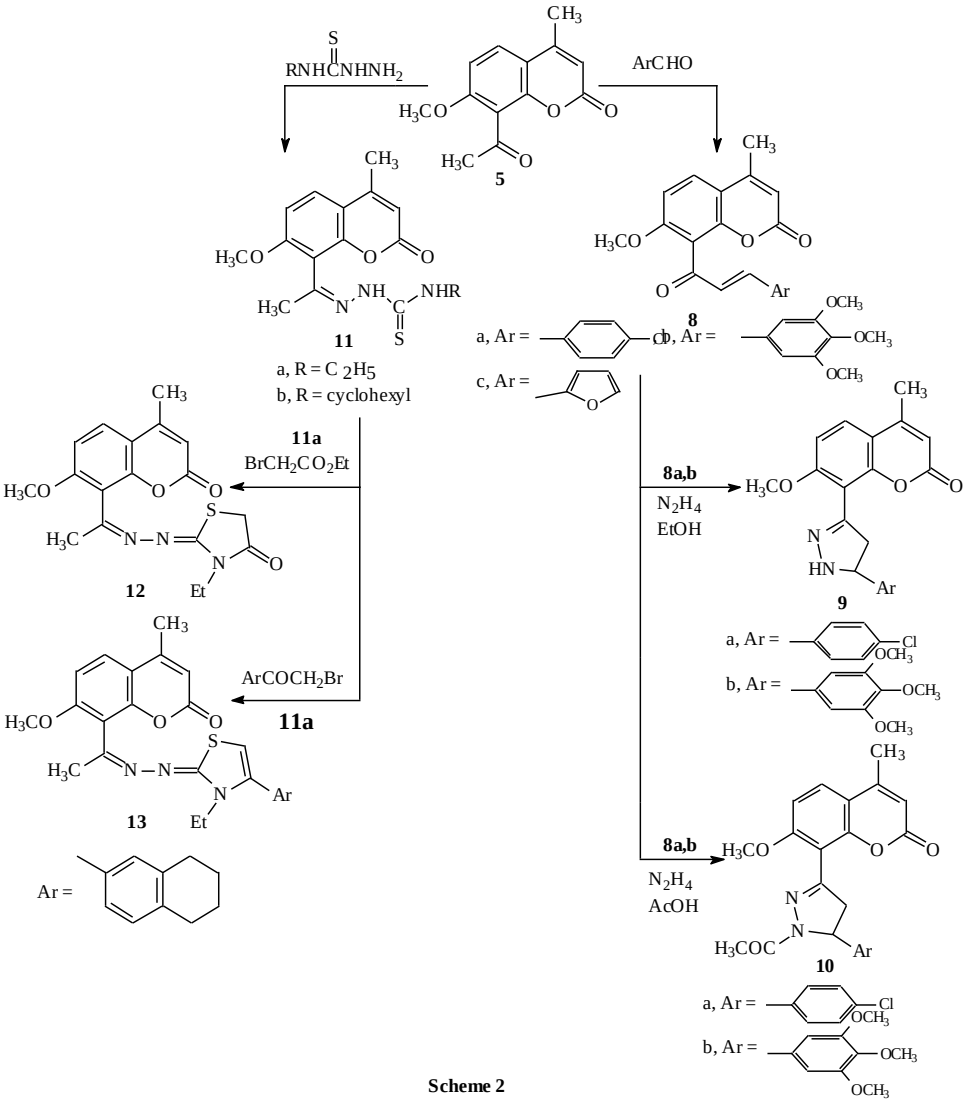
1- Synthesis of Coumarin derivatives:

The rationales for the synthesis and characterization of the selected coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), by elemental analysis, infrared, electronic spectra, room temperature magnetic measurements and powder X-ray diffraction were previously published by El-Zahar and El-Karim [22].



Scheme 1

*Cited from El-Zahar and El-Karim [22]



Scheme 2

Cited from El-Zahar and El-Karim [22]

Structure formula of the eight coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), selected from Schemes 1 and 2, are as follows**:**

(2): 7-Hydroxy-4-methyl-7[1-(phenylhydrazono)ethyl] coumarin

(3b):8-Acetyl-4-methyl-coumarin-7-yl-4-substitutedbenzene sulphonate

(5): 8-Acetyl-7-methoxy-4-methyl-coumarin.

(6b):**1,2-Dihydro-6-(7-methoxy-4-methyl-coumarin-8-yl)-2-oxo-4-substituted pyridine-3-carbonitrile.**

(8b): 7-Methoxy-4-methyl-8-(3-substituted) acryloyl) coumarin.

(10b):8-(1-Acetyl-5-(substituted)-4,5-dihydro-1H-pyrazol-3-yl)-7-methoxy-4-methyl coumarin.

(11a):4-Substituted-1-(1-(7-methoxy-4-methyl-coumarin-8-yl) ethylidene) thiosemi-carbazide.

(12): 7-Methoxy-4-methyl-8-[2-(3-ethyl-4-oxathiazolidin-2-ylidinene) ethylidin-hydrazono]coumarin.

2- Measurement of potential cytotoxicity by SRB assay

The selected coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), were subjected to a screening system for evaluation of their antitumor activity against Breast cancer MCF7 and liver HEPG2 cancer cell lines in comparison to the known anticancer drugs: 5-Flurouracil (5-FU) [20] and Doxorubicin (DOX) [21].

Potential cytotoxicity of the selected coumarin derivatives was tested using the method of Skehan *et al*. [23] as follows:

Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment to allow attachment of cells to the wall of the plate. Different concentrations of the compound under test (0, 1, 2.5, 5, 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated for 48 h at 37°C and in an atmosphere of 5% CO_2. Cultures were then fixed with trichloroacetic acid and stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl)aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader. The SRB assay results were linear with the number of cells and with values for cellular protein measured by both the Lowry and Bradford assays at densities ranging from sparse subconfluence to multilayered supraconfluence. The signal-to-noise ratio at 564 nm was approximately 1.5 with 1,000 cells per well. The relation between surviving fraction and drug concentration is plotted to get the survival curve of both cancer cell lines after the specified compound.

Biochemical Analysis

Animals

Male albino mice weighing 18-20 g were used in the present study. Mice were divided into three main groups as follows:

1- Group (1): Untreated or control group (5 mice).
2- Group (2): divided into two subgroups (5 mice for each subgroup) and treated with 5-FU or DOX as reference anticancer drugs.
3- Group (3): divided into eight subgroups (5 mice for each subgroup) and treated with the selected coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12).

Treatment

Group (1): each mouse was given a single intraperitoneal injection of 0.1ml DMSO.

Group (2): each mouse was given a single intraperitoneal injection of 0.1ml containing 12 mg/kg body weight 5-FU or DOX dissolved in sterile water.

Group (3): each mouse was given a single intraperitoneal injection of 0.1ml containing 12 mg/kg body weight of the selected coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12 respectively) dissolved in DMSO.

Blood was collected after 7 days from all mice groups.

The biochemical effects of the selected coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), on some liver enzymes such as aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP), were done using blood auto analyzer (Olympus AV 400, Japan) [24].

Moreover, albumin [25], globulins [26] and creatinine [27], total lipids [28], cholesterol [29], triglycerides [30] and bilirubin31 in serum of mice were evaluated in comparison to 5-FU and DOX.

Statistical analysis of the results was performed using Chi-square values (SPSS computer program).

Results and Discussion

Preliminary screening of the selected coumarin derivatives showed that all selected compounds exhibited a moderate to strong growth inhibition activity on the tested cell line between 1-10 µg/ml concentrations in comparison to the known anticancer drugs: 5-Flurouracil and Doxorubicin. Table (1) and Fig.(1 and 2) indicated the cytotoxic activity of the newly synthesized coumarin derivatives (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), against Breast cancer (MCF7) and liver HEPG2 cancer cell lines in comparison to the traditional anticancer drugs: 5-FU and DOX. It can be deduced from the results that compounds 8b, 11a, and 6b were the most active and induced a marked growth

inhibition, in a dose-dependent manner against MCF7 in comparison to DOX (IC_{50} equals 0.9, 1.28 and 1.94 µg/ml while DOX was 6.71µg/ml). On the other hand, compounds 6b and 11a, showed the highest effect against HEPG2 when compared to 5-FU and DOX (IC_{50} equals 1.02, and 1.88 µg/ml, while 5-FU and DOX were 5 and 3.56 µg/ml). However, the present data also indicated that all tested compounds gave lower growth inhibition effect against MCF7 in comparison to 5-FU (IC_{50} equals 0.67 µg/ml).

Table 1: Effect of selected coumarin compounds on breast MCF 7 and liver HEPG2 cancer cell lines

Compounds	**MCF 7 IC_{50}[µg/ml]**	**HEPG2 IC_{50}[µg/ml]**
5-Fluorouracil (5-FU)	0.67	5
Doxorubicin (Dox)	6.71	3.56
2	2.89	3.49
3b	3.15	8.63
5	3.36	4.47
6b	1.94	1.02
8b	0.9	2.62
10b	3.62	4.56
11a	1.28	1.88
12	3.09	3.55

IC_{50}: Dose of the compound which reduces survival to 50%.

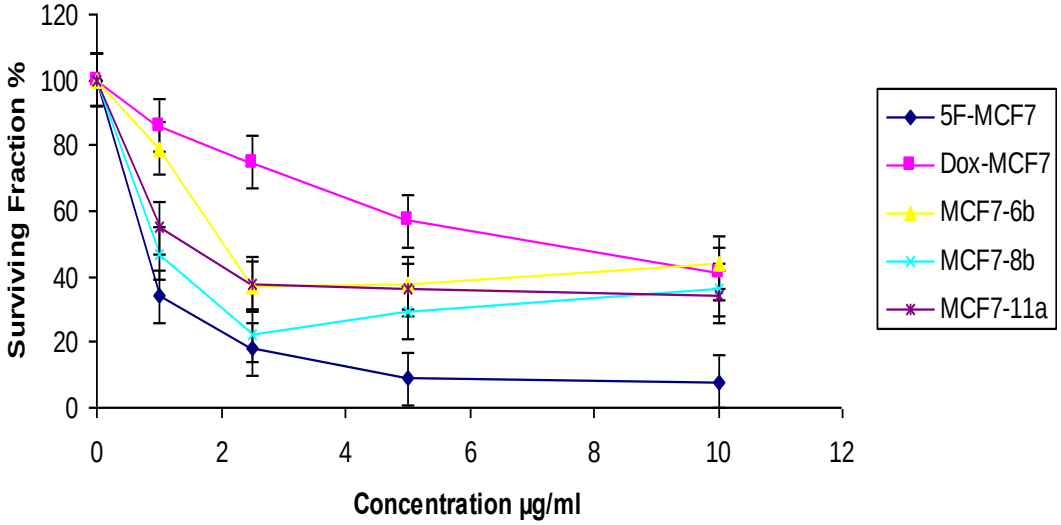


Figure 1: Cytotoxic Potency of the active compounds on MCF7 cell line

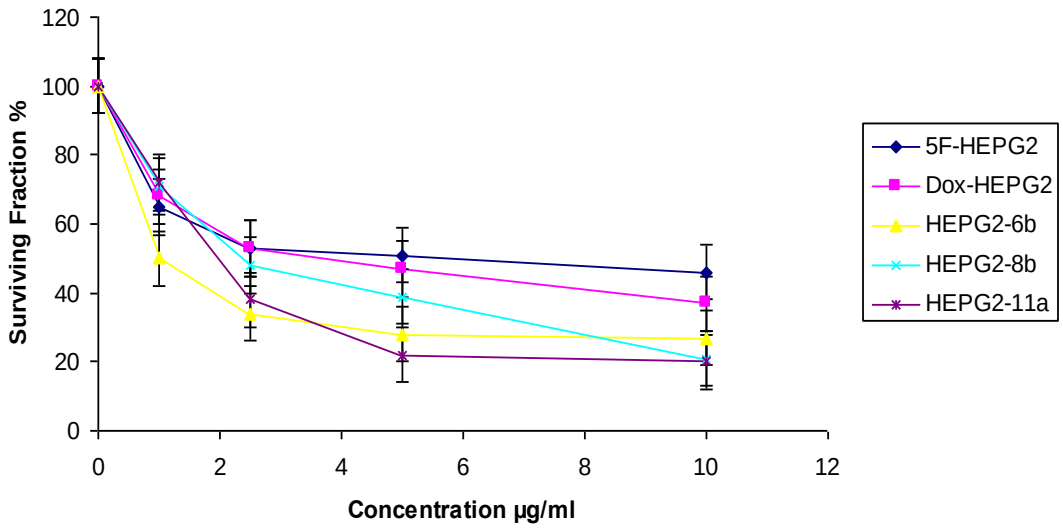


Figure 1: Cytotoxic Potency of the active compounds on HEPG2 cell line

Structure activity relationship

The cytotoxic potency of the eight different compounds were evaluated against MCF7. Table 1, showed that all compounds exhibited cytotoxicity, which indicates the importance of 4-methylcoumarin backbone for the activity. The replacement of the acetyl group of compound **5** (IC_{50} 3.36 µg/ml) with phenyl hydrazone side chain and free hydroxyl group slightly increased the activity compound **2** (IC_{50} 2.89 µg/ml). Also the insertion of phenyl sulphonyl group at 7-position of compound **3b** didn't exhibit significant change in the cytotoxicity compared to compound **5**, while the conversion of the acetyl group into pyridone nucleus compound **6b** led to high increase in the activity (IC_{50} 1.97 µg/ml).

Significant increase in the cytotoxicity was observed when a chalcone side chain was inserted compound **8b** (IC_{50} 0.9 µg/ml), while its cyclized derivative containing the pyrazoline moiety compound **10b** (IC_{50} 3.62 µg/ml) lost that increase to be near to that of start compound **5**.

The replacement of the acetyl group of compound **5** with thiosemicarbazone side chain increased the cytotoxicity significantly compound **11a** (IC_{50} 1.28 µg/ml), while the cyclization of the side chain to obtain the thiazolidinone moiety compound **12** (IC_{50} 3.09 µg/ml) decreased the activity again.

Effect of antitumour compounds on the biochemical parameters

Data obtained in table 2 presents the liver enzymatic activities (ALT, AST and ALP) in serum of control and treated groups of mice.

Table 2: Biochemical effects of 5-FU, Dox. and the coumarin derivatives on serum ALT, AST and ALP in mice.

Biochemical Parameters / Mice Groups	Alanine amino transferase Mean ± SD ALT (IU/ml)	Aspartate amino transferase Mean ± SD AST (IU/ml)	Alkaline phosphatase Mean ± SD ALP (k.k./dl)
Control	43.5 ± 2.03	108.32 ± 4.19	18.70 ± 1.10
5-FU	51.47 ± 9.02	130.431 ± 8.92	25.485 + 6.03
P<	0.001	0.001	0.001
Doxorubcin	59.26 ± 12.03	147.226 ± 16.34	30.317 ± 5.14
P<	0.001	0.001	0.001
2	46.21 ± 4.17	107.81 ± 4.25	21.94 ± 3.4
P<	n.s.	n.s.	0.01

3b	46.09±6.13	110.06 ± 8.91	18.76± 3.02
P<	n.s.	n.s.	n.s.
5	50.81±12.01	119±9.56	22.07±3.42
P<	0.01	0.01	0.01
6b	61.6±11.8	136.72±22.09	32.53±8.24
P<	0.001	0.001	0.001
8b	43.73±4.7	112.81±9.88	21.59±3.42
P<	n.s.	n.s.	n.s.
10b	73.09±14.2	140.09±31.01	30.41±9.22
P<	0.001	0.001	0.001
11a	39.56±6.7	112.54±12.7	19.94±4.35
P<	n.s.	0.01	n.s.
12	58.36±9.7	131.8±26.43	39.82±8.5
P<	0.001	0.001	0.001

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant

The results showed that the values recorded for AST and ALT were significantly higher ($P < 0.001$) with 5-Fu and DOX treated groups of mice than the control. On the other hand, treatment with the new compounds (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), caused inverse effects, where some values recorded for AST and ALT were non significant (n.s.) or slightly higher ($P < 0.01$) in comparison to control. Moreover, the recorded data showed that ALP activities were significantly increased ($P < 0.001$) with the treatment of 5-Fu and DOX, while there was no significant changes in ALP activities upon treatment with some of the new compounds (compounds 3b, 8b, and 11a).

Data listed in table 3 demonstrates the comparison between the levels of total lipids, cholesterol, triglycerides and bilirubin in serum of treated mice and the control group. It can be deduced from the present data that 5-FU and DOX caused a significant increase in the level of these parameters while treatment with the selected compounds (compounds 2, 3b, 5, 6b, 8b, 10b, 11a and 12), showed moderate or no significant changes.

Table 3: Biochemical effects of 5-FU, Dox. and the coumarin derivatives on serum total lipids, cholesterol, triglycerides and bilirubin in mice

Biochemical Parameters / Mice Groups	Total Lipids mg/dl	Cholestrol mg/dl	Triglycerides mg/dl	Bilirubin mg/dl
Control	323.41 ± 27.1	94.32 ± 13.5	108.7 ± 16.8	0.63± 0.04
5-FU	378.2±31.4	105.9±11.7	126.5±19.4	0.75± 0.10
P<	0.001	0.001	0.001	0.001
Doxorubcin	366.7 ± 6.10	109.3 ± 14.2	137.8 ± 17.10	0.81± 0.19
P<	0.001	0.001	0.001	0.001
2	317.4 ± 30.7	93.24 ± 19.53	116.23 ± 20.5	0.51± 0.08
P<	n.s.	n.s.	n.s.	0.01
3b	329.71 ± 21.5	97.48 ± 16.7	112.54± 17.8	0.53± 0.04
P<	n.s.	n.s.	n.s.	0.01
5	364.19±23.8	105.6± 17.4	119.8± 19.3	0.76± 0.15
P<	0.001	0.01	0.01	0.01
6b	367.52± 31.7	119.6± 23.8	127.8± 20.4	0.91± 0.1
P<	0.001	0.001	0.001	0.001
8b	326.32± 19.3	96.5± 19.4	114.6± 10.7	0.66± 0.08
P<	n.s.	n.s.	n.s.	n.s.
10b	371.23± 26.7	110.9± 31.2	152.6± 34.5	0.77± 0.2
P<	0.001	0.01	0.001	0.01
11a	331.63± 17.5	96.4± 10.5	118.6± 19.70	0.68± 0.11
P<	n.s.	n.s.	0.01	n.s.
12	368.71± 32.2	111.5± 16.9	92.3± 9.60	0.91± 0.4
P<	0.001	0.01	n.s.	0.001

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant

Table 4 represents a comparison between the levels of albumin, globulins and creatinine in serum of control and treated groups of mice. It is clear from the results in the table that there was a slight increase in the level of albumin and creatinine and globulins in the 5-FU and DOX treated groups while there were moderate or non significant changes in pretreated groups with the coumarin derivatives.

Table 4: Biochemical effects of 5-FU, Dox. and the coumarin derivatives on serum albumin, globulin, creatinine in mice.

Biochemical Parameters / Mice groups	Albumin mg/dl	Globulin mg/dl	A / G ratio	Creatinine mg/dl
Control	5.63 ±0.51	4.32 ± 0.9	1.3	0.69±0.03
5-FU	6.49±0.92	5.75±0.8	1.13	0.81± 0.06
P<	0.01	0.01	0.01	0.01
Doxorubcin	6.37 ± 0.85	5.91 ± 0.63	1.078	0.78± 0.04
P<	0.01	0.01	0.01	0.01
2	5.92 ± 0.82	5.12 ± 0.9	1.15	0.73± 0.04
P<	n.s.	n.s.	n.s.	n.s.
3b	5.53 ± 0.71	4.88 ± 1.01	1.13	0.67± 0.1
P<	n.s.	n.s.	n.s.	n.s.

5	7.73± 0.52	6.25± 0.82	1.23	0.84± 0.06
P<	0.01	0.01	0.01	0.01
6b	6.38± 0.41	6.37± 0.81	1.002	0.78± 0.09
P<	0.01	0.01	0.001	0.01
8b	5.66± 0.92	4.87± 0.73	1.16	0.87± 0.3
P<	n.s.	n.s.	n.s.	0.01
10b	10.22± 1.35	8.96± 0.91	1.14	0.72± 0.21
P<	0.001	0.001	0.01	n.s.
11a	5.97± 0.34	4.09± 0.63	1.46	0.65± 0.09
P<	n.s.	n.s.	n.s.	n.s.
12	6.91± 0.53	6.83± 0.90	1.01	1.53± 0.21
P<	0.01	0.01	0.001	0.001

Data are expressed as Mean + S.D.

P< 0.01: significant, P< 0.001: highly significant, n.s. : non significant

Cytotoxic drugs remain the main stay of cancer chemotherapy and are being administered with novel ways of therapy such as inhibitors of signals [31-32]. It is therefore important to discover novel cytotoxic agents with spectra of activity and toxicity that differ from those current agents [33]. It is well known that chemotherapy aims to destroy the cancer cells with various types of chemicals [34]. The substances used are supposed to target mainly the cancer cells and doses are calculated to minimize the collateral damage to surrounding tissues, which nevertheless occurs [35]. This kind of treatment increases the entropy of the organism, suppresses the immune system, and forms a toxic cell environment which may destroy surrounding healthy cells. So it is important to minimize curing doses to the least amount possible as well as trying to minimize the side effects of these drugs. For this novel derivatives of coumarin possessing a broader spectrum of antitumor activity and fewer toxic side effects than 5-Fu and DOX have been sought. The antitumor activities of such compounds were assessed against MCF7 and HEPG2 cancer cell lines in comparison to the traditional anticancer drugs: 5-Fu and DOX. Regarding the antitumor activity study, the selected compounds showed reasonable antitumor activity in comparison to 5-FU and DOX. Moreover, study of the induced biochemical parameters of the tested compounds in mice showed insignificant differences relative to the control group which indicates a moderate margin of safety for the selected compounds. Comparable to 5-FU and DOX, a dose augmentation of compounds 8b and 11a, searching for possible higher potency, seems, consequently, realizable without undesirable implications. Furthermore, the selected compounds have important potential advantages over 5-FU and DOX because of their lower toxicity and their ability to induce lower biochemical parameters. These results are in agreement with Campos *etal* [36] and Kamalakannan and Venkappayya [37], who reported that novel derivatives of 5-FU possessing a broader spectrum of antitumor activity and fewer toxic side effects than 5-FU.

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